NCI ETI Branch
Flow Cytometry Core Laboratory

Simultaneous PhiPhiLux G1D2, annexin V, 7-AAD and immunophenotypic labeling of mammalian cells.

Viable cells can be simultaneously labeled with **PhiPhiLux G1D2** (allowing the detection of functional caspase 3), **fluorochrome-conjugated annexin V** (detecting PS asymmetry in the plasma membrane, an early marker of apoptosis), **7-aminoactinomycin D** (7-AAD) (detecting increased membrane permeability associated with both apoptosis and necrosis) and one or more fluorochrome-conjugated antibodies. Our laboratory uses a dual laser flow cytometer (either a Becton-Dickinson FACSCalibur or FACSVantage) for the simultaneous detection of the PhiPhiLux reagent (which emits in the FITC range), allophycocyanin-conjugated annexin V (which is excited by a HeNe or diode laser emitting at 632 nm and emits at 660 nm), 7-AAD (excited at 488 nm and emitting at 670 nm) and a PE-conjugated antibody. Fluorochrome compatibility is good, although careful intralaser compensation is required for simultaneous use of PE and 7-AAD. Little or no interlaser compensation is required for APC and 7-AAD.

The PhiPhiLux and annexin V reagents are usually available in kit form, with buffers provided. This method does not make use of any of the buffers provided in these kits, but attempts to replicate the optimal reaction conditions in the buffer system used. Do not use the kit buffers for this method.

Materials.

- **PhiPhiLux G1D2** (available from OncoImmunin, Inc., Gaithersburg, MD). OncoImmunin makes a wide variety of fluorogenic enzyme substrates that become fluorescent upon cleavage. The PhiPhiLux system is designed to detect caspase 3 activity using the consensus substrate peptide DEVD. Several fluorochrome systems have been incorporated into this reagent; we use PhiPhiLux G1D2 since it is excited by a standard 488 nm laser and emits at a wavelength range similar to FITC. The PhiPhiLux reagents are provides in sealed aliquots at 10 μM and can be stored at 4°C prior to opening; once the ampule is opened, any remaining substrate should be stored at –20°C. Avoid repeated freezing and thawing.

- **Allophycocyanin (APC)-conjugated annexin V** (available from Caltag Laboratories,Burlingame, CA). Annexin V has been conjugated to a variety of fluorochromes. We use APC-conjugated annexin V for multicolor flow applications. APC-annexin V is sold by Caltag as a stock solution at 0.2 mg/ml.

- **7-aminoactinomycin D** (available from Sigma Chemical Co. and Molecular Probes). 7-aminoactinomycin D (7-AAD) is a DNA binding dye that excites at 488 nm and emits in the far red, with a peak at 670 nm. 7-AAD should be dissolved in EtOH at 1 mg/ml and stored at –20^{o}C. Solublized stocks are good for three months. Diluted stocks should be used as soon as possible.

- **Dulbecco's PBS** (containing calcium and magnesium).

Method

- If the cells of interest are in *in vitro* tissue culture, harvest them and transfer them to 12 x 75 mm tubes. Centrifuge at 800 g and *completely* decant the supernatant. Nearly complete removal of the supernatant is critical for the following steps; the amount of remaining sup should be as low as possible (less than 50 μl if possible). It is assumed that the cell medium is complete for normal cell growth (containing serum and other growth factors); if cells are obtained from clinical or other in vivo sources, they should be washed in complete medium (such as RPMI containing 10% FBS) prior to use.

- Tap each tube to resuspend the cell pellet in the remaining supernatant. Add 50 μl of the PhiPhiLux reagent to each tube and shake. The OncoImmunin kit directions emphasize that the PhiPhiLux reagent should be diluted as little as possible for maximum detection, hence the need for minimal sample supernatant. Incubate the tubes for 45 minutes at 37^{o}C.

- Remove the tubes from the incubator and add 5 μl APC-conjugated annexin V per tube (from 0.2 mg/ml stock Caltag stock solution). The cells should not be washed between PhiPhiLux and annexin V addition. Incubate the cells at room temperature for 15 minutes.

- If subsequent immunohenotyping is desired, place the tubes on ice for 5 minutes (still no washing). Add the fluorochrome-conjugated antibody of interest and incubate for the necessary time interval (usually for 30 minutes). It should be noted that the tubes still contain the PhiPhiLux and annexin V reagents in the original supernatant; try to add the antibody in as minimal a volume as possible so as to not disrupt PhiPhiLux concentration equilibrium and loading. If no immunophenotyping is desired, skip this and go on to the next step.

- Resuspend the cells in 3 mls Dulbecco's PBS and centrifuge at 800 g. PBS containing calcium is critical for this single wash step, since annexin

V will reversibly dissociate from PS moieties in the absence of divalent cations.

- Decant the supernatant and resuspend cells in a solution of 7-AAD at 5 μg/ml in Dulbecco's PBS. Allow the samples to sit at room temperature for 10 minutes, then analyze. All samples should be analyzed within 30 minutes of 7-AAD addition.

PhiPhiLux can be analyzed through a standard FITC filter. 7-AAD can be analyzed through a Cy5 filter (such as a 675 nm narrow bandpass). APC can be detected through a 660 nm narrow bandpass filter. A FACSCalibur equipped with the fourth color option is an ideal instrument for analysis.

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